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^{32}P-postlabelling analysis of DNA adducts in monocytes of smokers and passive smokers

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Summary. In a controlled study, ten male volunteers were subjected to different smoking and passive smoking conditions. After 60 h of strictly controlled nonsmoking, five smokers were exposed to mainstream smoke only, while five nonsmokers were exposed to the gas phase of environmental tobacco smoke (ETS). In a second experiment smokers were mainstream and ETS exposed, while nonsmokers were exposed to complete ETS. Blood was drawn before and after smoking and DNA adducts were analysed from blood monocytes by the ^{32}P-postlabelling assay, using the nuclease P1 enhancement method. We detected DNA adducts in monocytes of all probands. These adducts unrelated to smoking showed interindividual differences but only minor intraindividual changes in four samples of the same donor. After smoking interindividually variable additional adducts were visible in active smokers only. These smoking-related adducts had disappeared after 40 h of nonsmoking and reappeared again in three out of five smokers after the second smoking period. We conclude that smoking causes an interindividually variable pattern of DNA adducts in active smokers. These adducts disappear in less than 2 d, owing to the fast turnover of monocytes in the intravascular system. The effects described could not be observed in heavily exposed passive smokers.

Key words: Postlabelling assay – Smoking – Passive smoking – Monocytes

Introduction

Although a series of suspected carcinogenic substances have been found in cigarette smoke, there is still no definite answer as to which specific compounds are responsible for the increased risk of smokers in acquiring lung cancer [6]. In addition, there is a growing need to evaluate a possible carcinogenic risk in passive smokers, suggested by several epidemiological studies, although the extent of the effect remains to be investigated [16]. Since the covalent binding of xenobiotics or their metabolites to DNA is an important indicator for genotoxic stress [15], we studied the formation of DNA adducts in monocytes of smokers and passive smokers. The ^{32}P-postlabelling assay, being one of the most sensitive methods, seems to be especially suited for analysing the formation of DNA adducts resulting from the exposure to complex mixtures of unknown composition [17]. After pretreatment with nuclease P1 the nucleotides carrying a covalently bound adduct are selectively labelled with ^{32}P-phosphate. This allows the detection of PAH or bulky adducts with a sensitivity of a few molecules per genome [5]. Though several studies using the ^{32}P-postlabelling assay have shown a higher level of adducts in several tissues of smokers as compared to controls [12, 14], this has not yet been possible with human white blood cells [10, 11]. Here we used isolated monocytes, because these cells are metabolically active without pretreatment [1] and are therefore expected to reflect the formation of DNA adducts in vivo.

Materials and methods

Design of the study. Ten male healthy volunteers, five smokers (age 19–28) and five nonsmokers (age 23–29) refrained from smoking 60 h before the first blood sample was drawn. During the 6 d of the study the probands received a standardized diet, equal in amount and quality. Two different exposure conditions were applied: The third day smokers were exposed to mainstream smoke only and the fifth day to mainstream smoke and ETS; nonsmokers were exposed to the gas phase of ETS for 8 h on the third day and to complete ETS for 8 h on the fifth day. In the first experiment the five smokers had to smoke a total of 24 cigarettes (1 cig./20 min) and exhaled the smoke through an oneway-valve into the exposure

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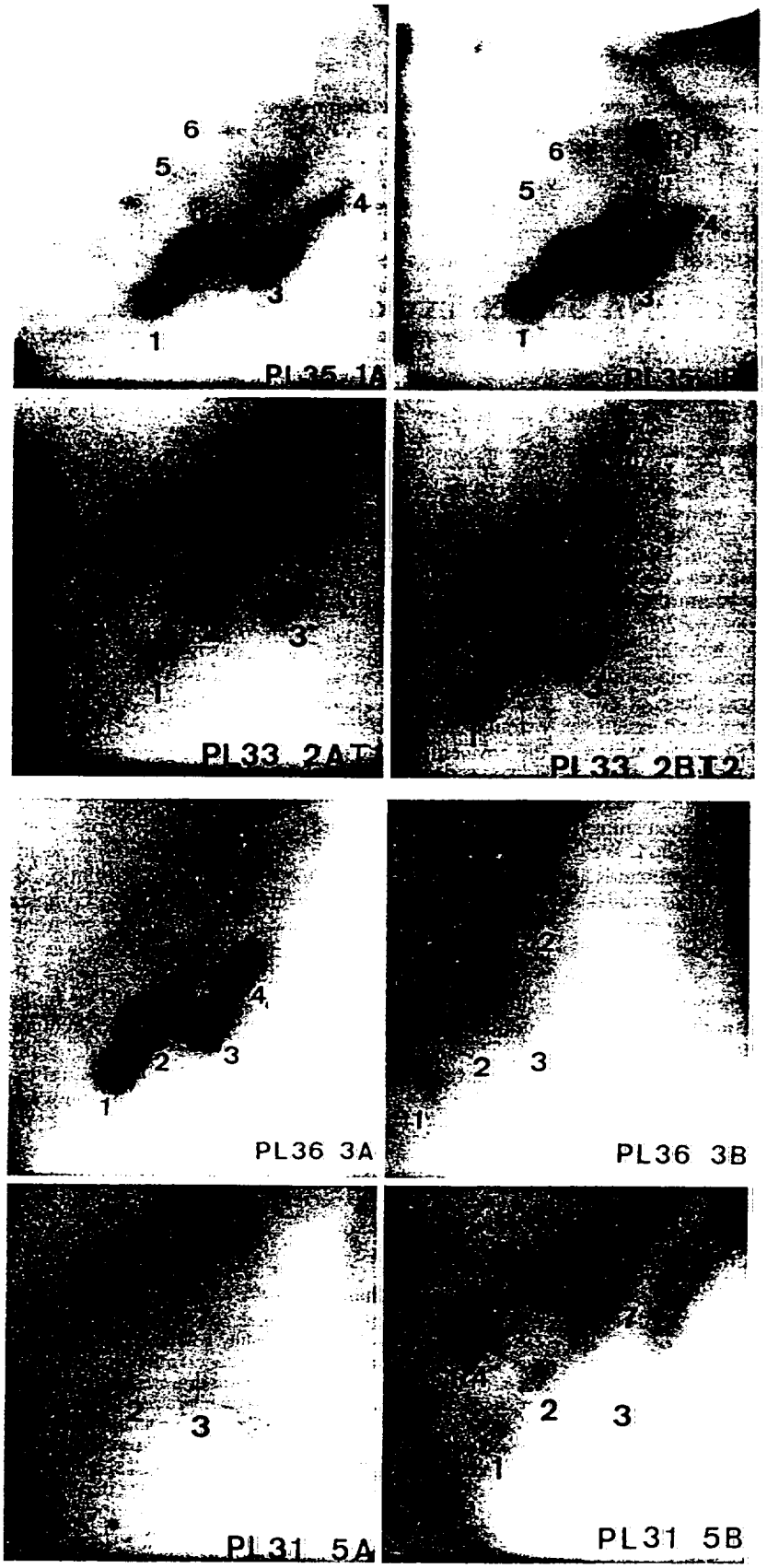

Fig. 1. Autoradiographs of PEI-cellulose t.l.c. maps of ^{32}P-labelled digests of DNA from peripheral blood monocytes of smokers (probands 1, 2, 3, 5). Sample A taken before and sample B taken after the first smoking period. R1, R2, R4, R5 are additional spots detected after smoking. Screen intensified autoradiography at −80°C for 72 h

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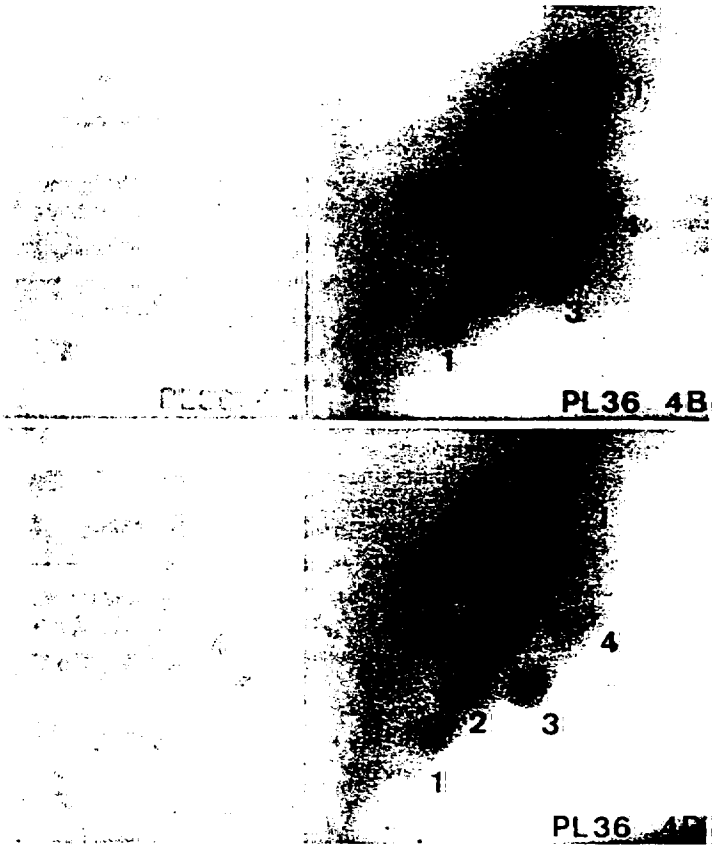

Fig. 2. Autoradiographs of PEI-cellulose t.l.c. maps of ^{32}P-labelled digest of DNA from peripheral blood monocytes of a smoker (proband 4). Samples taken before and after the first (A, B) and the second (C, D) smoking period. Smoking related adducts are only visible after smoking (R1, R2)

...al tobacco smoke ...oom had to carry ... and limited the ...ample A) and after ...were taken. After ...r equal conditions ...posed to the en... taken previous to ...mple D). Room air ...days and amounted ...nicotine and 3 to

...Blood (60 ml) was ...syringe by venous ...cially layered over ...ten) in sterile 10-ml ...g for 40 min. The ...ere collected and ...H Gibco, Grand Is... calf serum (FCS, ...g/l sodium bicar...erred into culture ...d at 37°C for 24 h ...here to allow the ... Floating lympho...er with the culture ...th isotonic saline, ...and stored frozen.

...sion was placed on ...μm) and the cells were lysed for 1 h at 37°C by an aqueous solution of sodium dodecyl sulfate (SDS, 20 mg/ml, Merck, Darmstadt, FRG), proteinase K (0.5 mg/ml, Merck) 0.05 *M* sodium phosphate and 0.05 *M* potassium phosphate. Subsequently the filters were washed with a 0.02 *M* Na_2EDTA solution, treated for 15 min with RNAse A (0.15 mg/ml, Boehringer Mannheim), and the remaining DNA was washed with a solution of 5 m*M* sodium phosphate and 5 m*M* potassium phosphate. Then the filters were cut into small pieces and DNA was dissolved in 2 ml H_2O by a short ultrasonic treatment. The amount of DNA was determined after incubation of an aliquot of the solution with diaminobenzoic acid and subsequent fluorescence spectrometry with an excitation wavelength of 408 nm and emission wavelength of 500 nm [2]. Calf-thymus DNA (Boehringer, Mannheim, FRG) was used as a standard.

^{32}P-postlabelling. DNA samples (2 to 8 μg DNA) were evaporated to dryness and digested overnight at 37°C in a total volume of 4.8 μl containing 0.25 U micrococcal nuclease (Sigma N-3758, Deisenhofen, FRG), 0.4 mU spleen phosphodiesterase (Boehringer, Mannheim, FRG), 17 m*M* sodium succinate and 8 m*M* CaCl. The digest was then treated with 4.8 μl of an aqueous solution of nuclease P1 (1 U, Sigma N-8630), sodium acetate (0.12 *M*) and $ZnCl_2$ (0.09 m*M*). The samples were incubated at 37°C for 1 h and the reaction was terminated by adding an aqueous solution (1.9 μl) of 0.5 *M* Tris base [11]. The DNA samples (5 μl) were then ^{32}P-labelled with 5 μl of a solution prepared by mixing 25 μl [Gamma-^{32}P]ATP (250 μCi, >5000 Ci/mmol, Amersham International), 7.5 μl T4 polynucleotide kinase 3'phosphatase free (67.5 U, Boehringer, Mannheim, FRG), 13.4 μl H_2O and 25 μl of buffer (200 m*M* Bicine, 100 m*M* $MgCl_2$, 100 m*M* dithiothreitol, 10 m*M* spermidine). After an incubation for 30 min at 37°C, potatoe apyrase (60 mU, Sigma A-6132) was added to terminate the reaction. The labelled nucleotides were

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resolved by 4-dimensional chromatography on PEI-cellulose t.l.c. sheets (Machery-Nagell Duren, FRG). The plates were first developed with 1 *M* sodium phosphate, pH 6.0, to wash polar adduct-free nucleotides on to a paper wick. The paper wick was removed and the plates were developed again in 3.5 *M* lithium formate, 8.5 *M* urea, pH 3.5, and after turning the plates 90° in 0.8 *M* lithium chloride, 0.5 *M* Tris-HCl and 8.5 *M* urea, pH 8.0. Subsequently the plates were developed in 1.7 *M* sodium phosphate on to a paper wick again to lower the background activity on the plates. The adducts were detected by screen intensified autoradiography for 72 h at −80°C (Xomat 5, Kodak, Rochester, NY, USA).

Results

Tobacco smoke exposure of the subjects is clearly reflected in significant increases in carboxyhemoglobin (COHb) after the 8-h exposure sessions. On Day 3 of the study, COHb increased by 7.1% in the active smokers and by 2.2% in the passive smokers. The corresponding increases for Day 5 were 7.7 and 2.6%.

The cells of all probands showed various DNA adducts which exhibited interindividual differences in amount and chromatographic behavior but were unrelated to smoking (sample A). After the first 8-h period of smoking additional DNA adducts appeared in samples of all smokers (sample B). These smoking related adducts varied individually (Fig. 1). In monocytes from sample C, (i.e. after a 40-h nonsmoking period), these additional adducts had disappeared, while the nonsmoking related adducts remained. After the second 8-h smoking experiment the additional adducts were detected again in samples of three smokers (Fig. 2). Nonsmokers exposed to ETS (passive smokers) did not show any additional adducts. There was no difference between samples taken after exposure to the gasphase only (sample B) and taken after exposure to total ETS (sample D).

Discussion

Smoking caused DNA adducts in peripheral blood monocytes of active smokers. Passive smoking did not lead to similar adducts. Since the samples were analysed using the nuclease P1 enhancement method it can not be concluded that passive smoking did not cause any DNA adducts at all. Nuclease P1 also dephosphorilates nucleotides carrying smaller and rather polar adducts, which for instance may arise due to tobacco specific nitrosamines. Therefore by the method used here, predominantly PAH derived or other bulky adducts are likely to be detected. Smoking-related DNA adducts in monocytes had already disappeared after a 40-h nonsmoking period. This is probably due to the relative short lifespan of blood monocytes ($t_{1/2}$ = 8 h) [7] in the intravascular system and might explain why other authors [10, 11] were not able to detect differences between smokers and nonsmokers on peripheral blood leucocytes. Studies on lung tissue [12] and mouse skin [13] have shown a rather long half life of bulky DNA adducts, so that is unlikely to attribute the observed rapid disapearence of smoking-related adducts to a fast reacting repair mechanism in human monocytes.

All samples exhibited adducts not related to smoking (adducts 4–7). Similar adducts were not observed in fibroblast DNA, probably because these cells are cultivated under standardized conditions, and because preexisting adducts were diluted by multiple cell proliferation. Monocytes seem to mirror the actual amount of genotoxic stress and showed DNA adducts level in almost all samples analysed in our laboratory. The adducts 1, 2 and 3 (Fig. 1), however, which were found in almost all samples analysed in this study could also be found in fibroblast DNA, while calf thymus DNA never showed these spots. Therefore we assume that culture conditions may have caused these adducts. In addition, since their provenance remains finally unexplained, we do not make any attempt here to speculate about variable intensities of these spots. Other adducts in monocytes unrelated to smoking, however, must be either rather fast developing adducts since monocytes display a fast turnover in blood, or may represent pre-existing adduct levels in monocyte stemcells. Both, smoking-related adducts and unrelated adducts exhibited intraindividual variations. This could be explained by genetically determined differences in the amounts of various enzymes being responsible for metabolic activation or detoxification of exogenous and endogenous substances in the cell [3, 4, 8, 9].

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